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SYNTHESIS AND CHARACTERIZATION OF CLICKABLE DENDRIMER HYDROGELS FOR OCULAR DRUG DELIVERY

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science in Biomedical Engineering at Virginia Commonwealth University

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ABSTRACT

SYNTHESIS AND CHARACTERIZATION OF CLICKABLE DENDRIMER HYDROGELS FOR OCULAR DRUG DELIVERY

By Jingfei Tian

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
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Director: Dr. Hu Yang, Associate Professor, Biomedical Engineering

Topical medication is a standard treatment for glaucoma. However, frequent dosing makes the therapy inconvenient and patient unfriendly. There is a great need to develop new topical formulations that provide long lasting noninvasive drug release. In this thesis, novel clickable dendrimer hydrogels for anti-glaucoma drug delivery were synthesized and characterized. Polyamidoamine (PAMAM) dendrimers have been widely applied for drug delivery. The physical characteristics they possess include monodispersity, water solubility, encapsulation ability, and a large number of surface groups. Polycationic PAMAM dendrimer G3 was surface modified with alkyne-PEG₅-acid and then reacted with polyethylene glycol bisazide (PEG-BA, 1100 gmol⁻¹) through click chemistry to form a cross-linked hydrogel. The resulting hydrogels were characterized in terms of mechanical properties, swelling, structural morphology, pH-dependent degradation, anti-glaucoma drugs (brimonidine tartrate and timolol maleate) release and cytotoxicity. To fully explore PAMAM dendrimers to make clickable hydrogels, polyanionic PAMAM dendrimer G4.5 was also surface modified with propargylamine to possess alkyne groups and successfully formed a hydrogel with PEG-BA. The work conducted in the thesis

shows that clickable dendrimer hydrogels were successfully developed and shown to possess desired properties for delivery of anti-glaucoma drugs.

CHAPTER 1 INTRODUCTION AND BACKGROUND

1.1 Glaucoma

Glaucoma is an intraocular pressure (IOP)-associated ocular neuropathy characterized by abnormal structural and functional features at the optic nerve head, neuroretinal rim, optic cup, retinal ganglion cell axons, lamina cribrosa and loss of visual field¹. Glaucoma can cause devastating and irreversible damage to optic nerves.

Glaucoma has been recognized as a primary cause of blindness globally^{2,3}. As reported in the blindness prevalence survey², it contributes to 12% of worldwide blindness. Glaucoma accounts for 4% permanent blindness in more than 2 million glaucomatous patients in the United States^{4,5}. In China, there are approximately 9.4 million of glaucoma patients at the age of 40 or older⁶. Population-based surveys^{7,8} show that, glaucoma, especially primary open-angle glaucoma, has destructive impacts. It is considered to be a top factor contributing to irreversible vision loss among black people in Africa⁹⁻¹¹ and African Americans¹².

Glaucoma can be classified into two major types: primary glaucoma and secondary glaucoma.

Primary glaucoma is diagnosed as the group of glaucomatous disorder without preexisting systemic or ocular diseases. Secondary glaucoma, in the contrary, refers to glaucomatous exfoliation¹³, iridocorneal endothelial syndrome (ICE) syndrome¹⁴, disorder that is associated with ocular or systemic comorbidity such as eye injury, eye tumors, systemic disease like diabetes¹⁵, and so on.

Based on anatomical features at trabecular meshwork, the drainage pathway of aqueous humor¹⁶, glaucoma can also be grouped into open-angle glaucoma and closed-angle glaucoma. Open-

angle glaucoma has normal iridocorneal angle appearance, while the iridocorneal angle of closed-angle glaucoma is physically blocked by periphery tissue.

Elevated IOP has long been the research focus of the etiology of glaucoma. A survey shows that the IOP of 48% to 80% of patients are above the normal value, usually estimated at 21 mm Hg¹⁶. Even though glaucomatous vision loss is not necessarily associated with IOP, the risks of glaucoma development rise with a high level of IOP¹⁷. Primary open angle glaucoma accounts for 90% of the disease. However, its pathophysiology remains unclear. Closed-angle glaucoma occurs when aqueous humor cannot flow through the pupil into the anterior chamber (pupillary block). This situation causes pressure increase behind the iris and deforms the iris anteriorly (iris bombe), and thus mechanically blocks the drainage pathway and prevents aqueous humor outflow through the trabecular network¹⁶.

A number of risk factors have been recognized for open-angle and closed-angle glaucoma including advancing age, family history¹⁸, history of ocular trauma¹⁹, hypertension and diabetes¹⁵, eye injury, eye tumors, ICE syndrome¹⁴, vasospastic diseases, and a reduction in blood circulation to the optic nerve. Besides, there exist ethnic-dependent variations in the prevalence of glaucoma. In particular, the prevalence of open-angle glaucoma appears to be threefold more in African Americans than in Caucasians in the United States^{12,20}. Furthermore, primary open-angle glaucoma is the major cause of blindness among African-Americans¹². In contrast, Asians are more likely to develop closed-angle glaucoma²¹.

1.2 Glaucoma management

As of today, lowering elevated IOP to normal range remains a primary treatment strategy worldwide. To prevent vision loss, eye surgeries (like laser trabeculoplasty, iridotomy,

iridectomy, canaloplasty and trabeculectomy) can be performed to enhance aqueous humor drainage. However, success of surgeries depends on surgical skills and eye responses of patients. In addition, glaucomatous surgery may lead to fibrosis formation, which is a risk factor for cataract. To overcome disadvantages and limitations of surgery, medication is widely adopted for glaucoma management.

1.2.1 Drug delivery

Anti-glaucoma agents in common clinical use include prostaglandin analogs, parasympathomimetic (miotic) agents, including cholinergic and anticholinesterase agents, carbonic anhydrase inhibitors, adrenergic antagonists, α_2 agonists, hyperosmotic agents and so on²²⁻²⁵. According to chemical structure and pharmacologic effects, medications are divided into different groups (Table 1-1).

Usually, the combination of anti-glaucoma drugs from different groups may have better treatment outcomes. For instance, adrenergic agent brimonidine tartrate and beta blocker timolol maleate are often combined to make a better control over IOP. To this end, brimonidine tartrate and timolol maleate are used as model drugs in this thesis.

Table 1-1 Summary of mechanism and adverse effect of different types of anti-glaucoma drugs (adapted from²⁶).

Category	Name	Mechanism of action	Adverse effects
Prostaglandin analogs	Latanoprost (Xalatan)	Increased USO (uveoscleral outflow)	Pigmentation of eyelashes, eyelid skin pigmentation, hyperemia (red eye), flu-like symptoms (joint/muscle pain and headache)
	Bimatoprost (Lumigan)	Increased USO (uveoscleral outflow)	Blurred vision, eyelid redness, eye discomfort, permanently darken iris, darken/thicken eyelashes
	Travoprost (Travatan)	Increased USO (uveoscleral outflow)	Blurred vision, eyelid redness, eye discomfort, permanently darken iris, darken/thicken eyelashes
Beta blockers	Timolol	Decrease aqueous production by ciliary body	Bronchospasm, bradycardia, depression, impotence
	Betaxolol	Decrease aqueous production by ciliary body	Fewer pulmonary complications due to selective Beta blockage
	Levobunolol (Betagan)	Decrease aqueous production	
Adrenergic agents	Brimonidine	Decrease aqueous production, increase USO	Blurring, foreign body sensation, eyelid edema, dryness, headache, fatigue, hypotension, depression, insomnia
	Aproclonidine	Decrease aqueous production, increase USO	
Miotics	Pilocarpine	Increase trabecular outflow by contraction of the ciliary muscle, opening the trabecular meshwork	Posterior synechia, keratitis, miosis, brow ache, cataract, myopia, retinal tear, dermatitis, increased salivation
Carbonic anhydrase inhibitors	Dorzolamide	Decrease aqueous production by inhibiting carbonic anhydrase in the ciliary body	Eye irritation, bitter taste
	Brinzolamide	Decrease aqueous production by inhibiting carbonic anhydrase in the ciliary body	Eye irritation, bitter taste
	Acetazolamide	Decrease aqueous production by inhibiting carbonic anhydrase in the ciliary body	Malaise, depression, weight loss, kidney stones

Ocular drug delivery is challenging and complicated due to the presence of various anatomical and physiological barriers. Depending upon administration routes such as topical administration, systemic administration and injection, delivery drugs may encounter different barriers. Table 1-2 summarizes these specific drug delivery barriers facing various administration routes.

Table 1-2 Administration routes and anatomical and physiological barriers of ocular drug delivery (adapted from²⁷)

Administration route		Anatomical and physiological barriers
Topical administration		Cornea, sclera and conjunctiva
Systemic administration		Blood-aqueous barrier and blood-retina barrier, targeted ocular tissue
Injectable administration	Periocular administration	Dynamic including conjunctival blood and lymphatic circulation static, and metabolic barriers
	Intravitreal administration	Non-uniform drug distribution in vitreous, vitreous is barrier for retinal gene delivery

1.2.1.1 Hydrogel

Hydrogels are three-dimensional crosslinked polymer networks that can absorb substantial amounts of aqueous solutions. Due to their high water content, hydrogels bear resemblance to natural tissue more than other types of synthetic biomaterial. And this unique property of hydrogel has sparked particular interests in drug delivery application. A variety of polymers such as PLA, PLGA²⁸, chitosan²⁹ and dendrimer³⁰ can be utilized to synthesize hydrogels. Among them, PAMAM dendrimers have been widely applied for drug delivery due to their beneficial physical characteristics include monodispersity, water solubility, encapsulation ability, and a large number of functional groups on the surface. Thus, using dendrimers as building blocks to make hydrogels can integrate the unique characteristics and structural features into the hydrogel network.

Hydrogels can be made by using a variety of methods. In general, they are divided into non-covalently crosslinked methods and covalently crosslinked methods. As a branch of covalent chemical reaction, click chemistry, first described by K. Barry Sharpless and Valery Fokin in 2001^{31,32}, has been applied to many research fields including biomaterial and drug delivery^{33,34}. As a highly efficient chemistry method, it has also been applied to hydrogel synthesis.

1.2.1.2 Contact lens

Drug-eluting contact lens can be generated by various methods including soaking of lenses in drug solution, copolymerization of the contact lens hydrogels with functional monomers (which provide binding points for drugs and communicate functionality to the contact lens), dispersion or immobilization on the contact lens surface of drug-loaded colloidal nanoparticles, molecular imprinting and supercritical solvent impregnation method³⁵.

After a drug-eluting contact lens is placed on the eye, a normal human tear film, about 7-10 μm in thickness, partitions into two parts: pre-lens tear film and post-lens tear film³⁶. The drug-eluting contact lens then releases drug into the pre-lens tear film and the post-lens tear film. The drugs released into the pre-lens tear film are likely to be absorbed into the conjunctiva or drained through the canaliculi. They eventually enter the systemic circulation. However, drugs released into the post-lens tear film will either be transmitted through the cornea or be diffused radially out into the outer tear lake. Resulting from the very large discrepancy of the post-lens tear film in the thickness ($\sim 5 \mu\text{m}$) and contact area with a radius of 5 mm, almost the entire drug amount released into the post-lens tear film diffuses into the cornea³⁷.

Scientists have tried to apply contact lens to glaucoma treatment³⁷⁻³⁹. Nevertheless, a majority of the current relative researches are only performed to evaluate therapeutic contact lens in vitro. In

vivo evaluation of contact lens in ocular drug delivery has not been widely and deeply performed and deserves further investigation.

1.2.1.3 Liposome

First studied in 1981, Smolin and coworkers⁴⁰ applied liposomes in ocular drug delivery. Since then, scientists have been trying to fulfill the potential of liposomes in ocular drug delivery in various approaches⁴¹ and have had great progresses.

A liposome composed of a lipid bilayer is an artificially-prepared vesicle. It encapsulates an aqueous region inside a hydrophobic membrane. Due to this biphasic nature, both lipophilic and hydrophilic drugs can be encapsulated in the liposome. If the drug is water soluble, it will be entrapped in the aqueous region. If the drug is hydrophobic, it will associate with the hydrophobic membrane or dissolve in the lipid bilayer⁴². In drug delivery process, drugs or gene therapy can be delivered into the cell after the lipid bilayer fusing with the cell membrane. In the case of topically applied liposomes, viscosity inducing agents is indispensable in the way of providing a consistency which is easy to apply and has better patient acceptability⁴³. However, despite of many advantages including nano-scaled size, better biocompatibility, biodegradability, stability, sustained drug release and toxicity reduction, its disadvantages existing in aspects of low loading and manufacturing difficulties⁴⁴ obstruct its way to be made into marketed products.

1.2.2 Eye drop

Eye drop is the most common and conventional form of topical medication for glaucoma treatment. However, the effectiveness of eye drop is compromised by several drawbacks such as low drug penetration, low bioavailability, poor patient compliance, frequent dosing requirement etc. Typically less than 5% of drugs in eye drops can be successfully delivered to the ocular

tissues of the anterior chamber and almost negligible quantities to the posterior chamber^{45, 46}. To overcome those deficiencies, new and effective drug delivery formulations such as drug-eluting contact lens, liposome-based drug delivery vehicles, hydrogels, nanocarriers and implant devices are under development.

1.2.3 Gene therapy

Gene therapy is a neuroprotective approach aiming at optic nerve regeneration and regaining of vision. Given that the slow death of retinal ganglion cells (RGCs) and their axons account for progressive vision loss and permanent blindness⁴⁷, gene therapy primarily focuses on regeneration of RGCs. There are several theories that support this approach. Mechanical theory postulates that decreased neurotrophic factor (NF) production in retinal ganglion cells due to elevated IOP triggers retinal ganglion cells apoptosis. In the meanwhile, increased IOP obstructs the bidirectional axonal flow of NF and proteins made by RGCs⁴⁸ between RGCs, leading to axonal damage⁴⁹. The vascular theory, however, associates deterioration of RGCs with abnormally low ocular blood flow, which is postulated to play a major role in the production of free radicals and consequent oxidative stress on RGCs^{50, 51}.

It was found that NF, especially brain-derived neurotrophic factor (BDNF)⁵², ciliary neurotrophic factor (CNTF)^{53, 54} and glial cell line-derived neurotrophic factor (GDNF)⁵⁵ increase the survival of RGCs.

In general, gene delivery system can be divided into two types based on types of carriers. Viral gene delivery utilizes genetically modified viruses to deliver therapeutic gene. Nonviral gene delivery typically employs synthetic carriers like polymeric and lipidic macromolecules to deliver genetic therapeutics⁵⁶. In addition to RGC, other specific tissues contribute to glaucoma

formation including the trabecular meshwork, ciliary body and ciliary epithelium. A recent study showed the success of gene expression mediated with intracameral injection of a lentiviral vector for as long as 15 months in the trabecular meshwork, iris and ciliary body of nonhuman primates⁵⁷.

1.3 Click Chemistry

Click chemistry is a group of chemical reactions which meet many criteria. The desirable click chemistry reaction should be modular, stereospecific, wide in scope, give very high yields, and generate only inoffensive byproducts³¹. The process would preferably have simple reaction conditions, use readily available starting materials and reagents and use no solvent or use a solvent that is benign or easily removed (preferably water), provide simple product isolation by non-chromatographic methods. It is unlikely to find such a reaction that perfectly fits the criteria. However, several reactions have been proved to fit the criteria better than the others. Among them, discovered by Rolf Huisgen, the Huisgen 1, 3-dipolar cycloaddition⁵⁸ (Figure 1-1) is the most popular one and has recently gained a considerable amount of attention.

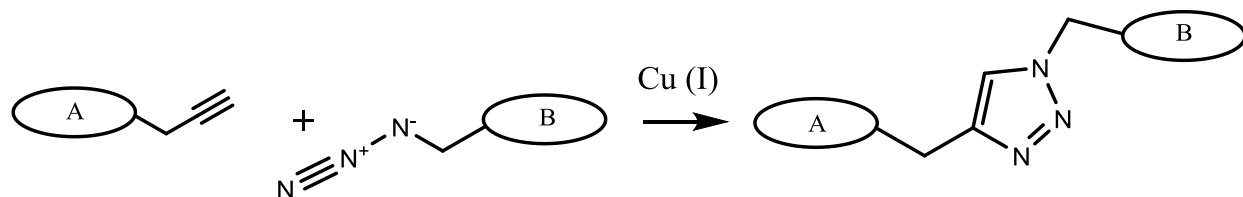


Figure 1-1. Schematic of azide-alkyne Huisgen cycloaddition. Catalyzed by Cu (I), azide and alkyne react to give a 1, 2, 3-triazole.

In solution, alkyne groups and azide groups are quietly inert. However, they can react with each other and form a triazole immediately as the catalyst is added. Cu (I) is gained by reducing Cu (II) oxidation state to Cu (I) by adding a reducing agent. The advantage of this method over directly

adding Cu (I) compound is that the presence of reducing agent makes up for any oxygen which may have gotten into the system. Oxygen oxidizes the Cu (I) to Cu (II) which impedes the reaction and results in low yield. The most commonly used Cu compound is copper sulfate. And sodium ascorbate is the most commonly used reducing agent.

1.4 Dendrimer

Dendrimers are a group of highly branched, symmetric spherical macromolecules. Due to a number of unique features: nanoscale size, monodispersity, manipulable surface modification, water-solubility and multivalency, they have been widely applied to drug delivery research. PAMAM dendrimers, composed of an ethylenediamine (EDA) or an ammonia core with methyl acrylate and ethylene diamine branches, have been the most investigated in dendrimer family for drug delivery. The properties of PAMAM dendrimer are dominated by surface functionality. Full generations (cationic) have amine surface groups and half generations (anionic) have carboxyl surface groups. To deliver drugs, drugs can be physically encapsulated in dendrimers or chemically conjugated to dendrimers.

CHAPTER 2 MATERIAL AND METHOD

2.1 Material and reagent

Table 2-1 List of materials and reagents and abbreviations

Material	Abbreviation
4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride	DMTMM
Alkyne-PEG ₅ -acid	
Brimonidine tartrate	BT
Cell proliferation reagent WST-1 assay	
Copper(II) sulfate pentahydrate	CuSO ₄ ·5H ₂ O
Dialysis tubing (MWCO 3500)	
Dimethylformamide	DMF
Ethylenediaminetetraacetic acid	EDTA
Generation 3 polyamidoamine dendrimer with core of ethylenediamine (EDA)	G3
Generation 4.5 polyamidoamine dendrimer with core of ethylenediamine (EDA)	G4.5
Human corneal epithelial cells	HCECs
Human IL-1 α ELISA kit	
Hydrochloric acid	HCl
Phosphate buffer solution (of pH 4.5, 5.5 and 7.4)	PBS
Poly(ethylene glycol) bisazide (MW=1100 gmol ⁻¹)	PEG-BA
Propargylamine,	
Sodium ascorbate	
Sodium Bicarbonate	NaHCO ₃
Timolol maleate	TM
Azide-PEG ₅ -acid	

2.2 Equipment

Table 2-2 List of equipments

List of equipment	Function
3500 MW Dialysis Tubing	For hydrogel purification and weight specific elution of drug into filtrate
Flexi-Dry FTS System	Freeze dry system to dry frozen samples
Hot Water Bath	To provide 37°C aqueous environment to simulate drug release in physiological conditions in vitro
Incubator	Temperature and humidity control of cell culture
MTS Bionix 200 ® Mechanical Testing System	Measure mechanical properties of materials
Rotary Evaporator, Heidolph LABOROTA 4000	Distillation of low boiling point chemicals from mixture of compounds
Ultra Violet Visible (UV-Vis) Spectrophotometer	Quantitative tool using light absorption to measure the amount of sample released
Varian Mercury-300 MHz Nuclear Magnetic Resonance (NMR) spectrometer	Measuring chemical shifts of protons, ¹ H-NMR
Weighing Balance	Used to measure mass of materials needed
Scanning Electron Microscope (JEOL LV-5610)	Obtain high resolution images of hydrogels for morphology characterization
Zeiss Invertoskop 40C Microscope	Examining cell growth and for cell counting

2.3 Experimental methods

2.3.1 Synthesis of clickable cationic PAMAM dendrimer hydrogel

2.3.1.1 Synthesis of PAMAM G3-alkyne conjugates

To a solution of 40mg of dendrimer G3 in 1mL of 0.1M NaHCO₃, 84.6mg of DMTMM was added. The solution of 84.6 mg of alkyne-PEG₅-acid in 500μL of DI water was added dropwise. The molar feed ratio of amine: alkyne: DMTMM is 1:1.5:1.65. The reaction mixture was stirred overnight at room temperature. Product was purified by dialyzing against DI water and then freeze-dried to obtain G3-alkyne conjugates. G3-alkyne conjugates were characterized using Varian Mercury-300 MHz NMR.

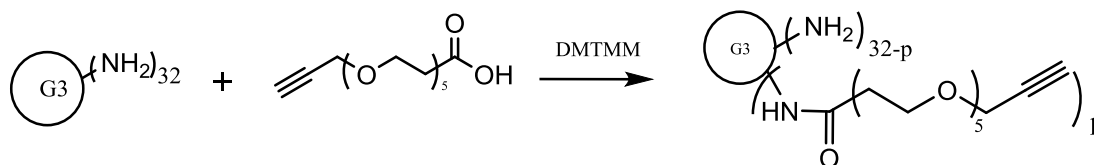


Figure 2-1 Synthesis of G3-alkyne conjugates.

2.3.1.2 G3 hydrogel synthesis and anti-glaucoma drug encapsulation

53.9 mg of PEG-BA was dissolved in 533 μl DI water, and then mixed with 40.0mg of G3-alkyne. The mixture was vigorously stirred and then evenly divided to 3 vials: control group, BT group and TM group. Drug-loaded hydrogels were prepared by adding 4.0 mg of brimonidine tartrate to BT group and 4.0 mg of timolol maleate to TM group. 2.7 mg of CuSO₄·5H₂O was added to resolve in each group. The vials were vigorously stirred for 30 seconds and hydrogels formed instantly upon addition of 3.8 mg ascorbic acid to each group. The molar ration of alkyne: azide: Cu: sodium ascorbate was 1:1.5:0.5:1.

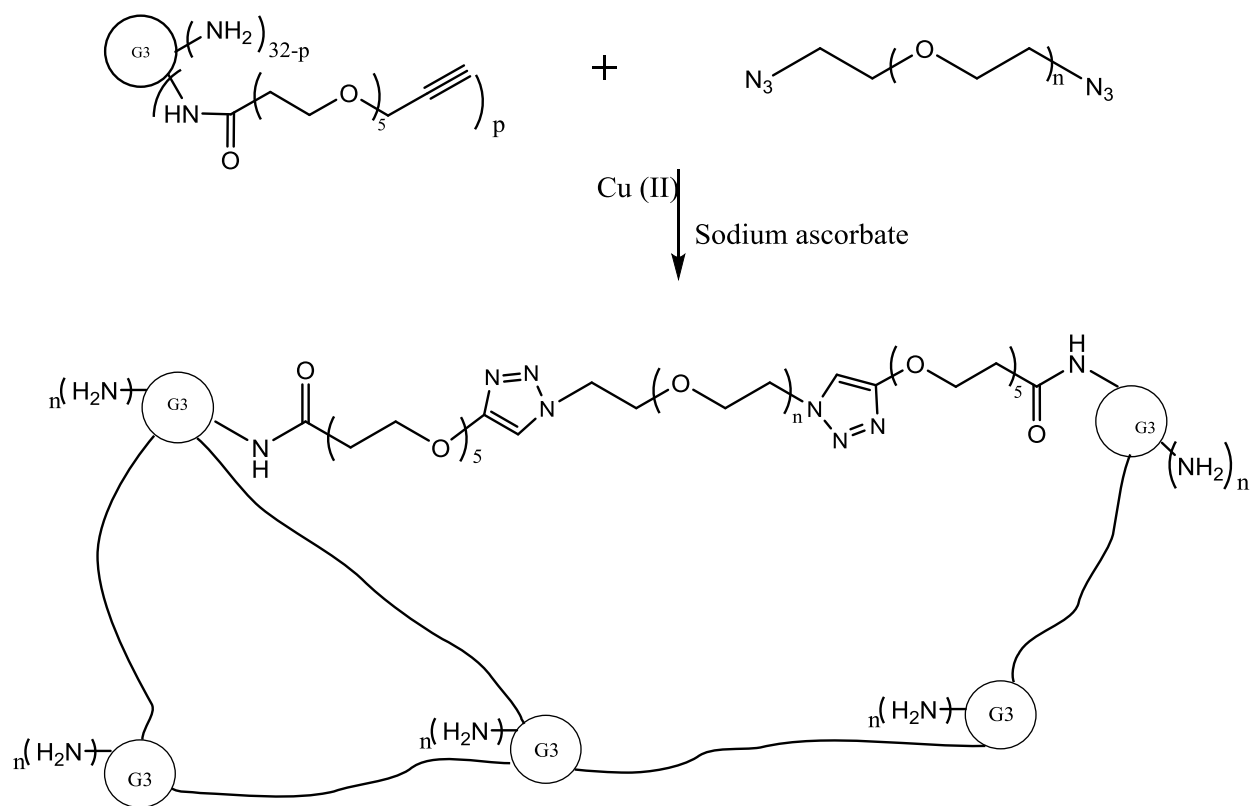


Figure 2-2 Synthesis of clickable cationic PAMAM dendrimer hydrogel on the basis of G3-alkyne and PEG-BA.

2.3.2 Synthesis of clickable anionic PAMAM dendrimer hydrogel

2.3.2.1 Synthesis of PAMAM G4.5-alkyne conjugates

To a solution of 50.0 mg PAMAM dendrimer G4.5 in 3.5 mL of 0.1M NaHCO₃, 84.0 mg of DMTMM was added. The solution of 15 μ L of propargylamine in 500 μ L DMF was added dropwise. The reaction mixture was stirred overnight. Upon removal the solvents under reduced pressure, the remaining residue was dialyzed against DI water and freeze-dried to obtain G4.5-alkyne conjugates. G4.5-alkyne conjugates were characterized using Varian Mercury-300 MHz NMR.

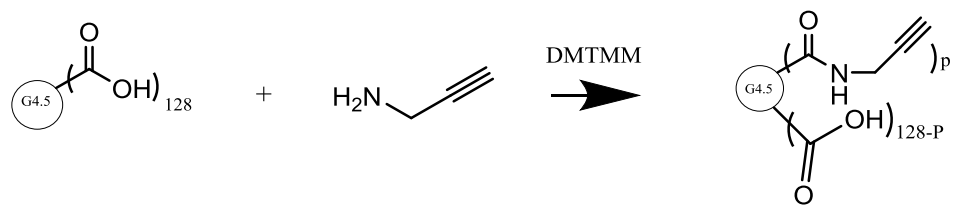


Figure 2-3 Synthesis of G4.5-alkyne conjugates.

2.3.2.2 Determination of alkyne in G4.5

8.0 mg of G4.5-alkyne conjugate was dissolved in 1.4 mL DI water, and then mixed with 12.1 mg of azide-PEG₅-acide. The mixture was vigorously stirred. 4.5mg of CuSO₄·5H₂O was added to resolve in the solution. The solution of 7.2 mg of sodium ascorbate in 100 μ L of DI water was added to the stirring system dropwise. The molar ration of G4.5-alkyne conjugate: azide: Cu: sodium ascorbate was 1:128:64:128.

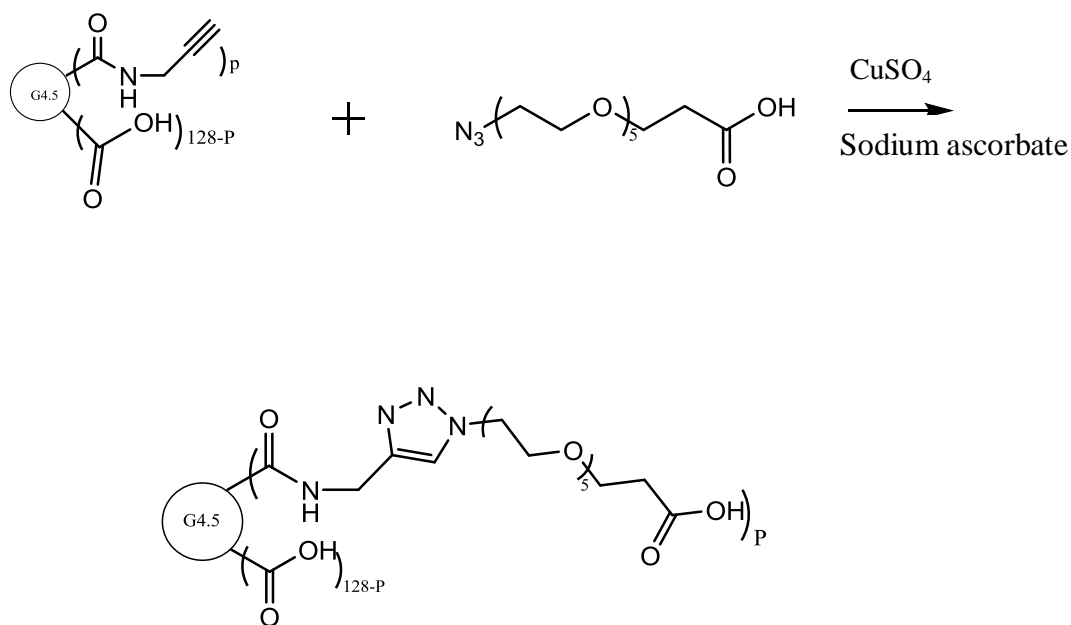


Figure 2-4 Schematic Representation of the reaction between G4.5-alkyne and N₃-PEG₅-CH₂CH₂COOH.

2.3.2.3 G4.5 hydrogel synthesis

17.2 mg of PEG-BA was dissolved in 200 μ l of DI water, and then mixed with 50.0 mg of G4.5-alkyne. The mixture was vigorously stirred. Clickable hydrogel formed immediately upon sequential addition of 2.6 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 3.7 mg of sodium. The molar ratio of alkyne: azide: Cu: sodium ascorbate was 1:1.5:0.5:1.

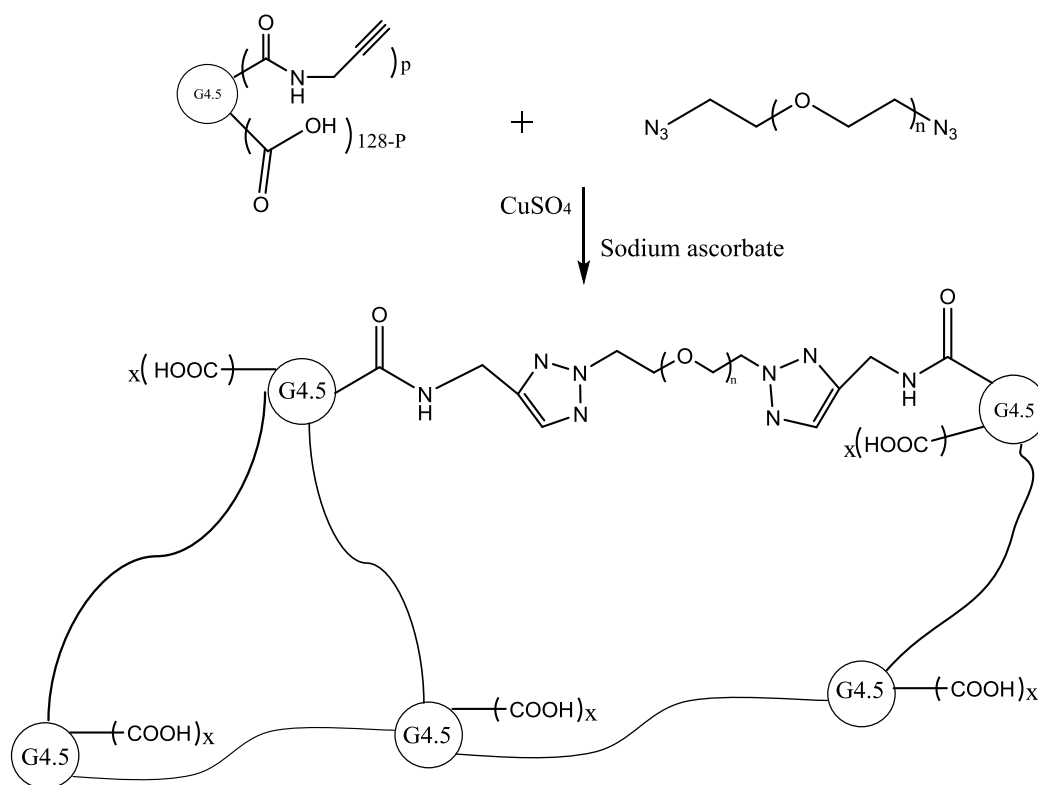


Figure 2-5 Synthesis of clickable anionic PAMAM dendrimer hydrogel.

2.3.3 Hydrogel purification

Hydrogels were put into dialysis tube (MWCO 3500), then purified by dialysis against 1mM Ethylenediaminetetraacetic acid (EDTA) for 2 days. Dialysis against DI water for two days was further done to remove EDTA.

2.3.4 Characterization

2.3.4.1 ¹H NMR spectroscopy

¹H NMR spectra of were collected on a 300 MHz NMR spectrometer (Mercury-300). The solvent used was deuterium water (D₂O), which has a chemical shift of 4.79 ppm.

2.3.4.2 Mechanical property study

The mechanical properties of G3 hydrogels were tested in terms of compressional property by using MTS Bionix 200 ® Mechanical Testing System and TestWorks 4.0 software. The three air-dried G3 hydrogels were 1.15mm, 1.09mm and 1.11mm at its width, and 1.47mm, 1.41mm and 1.58mm at its thickness respectively. Mechanical properties of the three scaffolds including peak load, peak stress and modulus were obtained.

2.3.4.3 Swelling degree measurement

Three air dried hydrogel samples were immersed into DI water at room temperature. At pre-determined time intervals, samples were taken out, blot dried and weighed. The experiment stopped when the sample reached a stable weight. The swelling degree was calculated using the following equation:

$$\text{Swelling degree (\%)} = \frac{w_s - w_d}{w_d} \times 100,$$

where w_s is the weight of the swelling hydrogel and w_d is the weight of dry hydrogel.

2.3.4.4 PH-dependent hydrogel degradation study

The in vitro degradation of hydrogels was evaluated in 37 °C phosphate buffered saline (PBS) of three different pHs (pH4.5, 5.5 and 7.4). After air-dried and weighed, samples were individually immersed in a centrifugal vial with 1.2 ml of one of the three solutions and incubated at 37°C in a thermostatic water tank. The sample residues were taken out, air-dried and weighed every 24 hours for five days. The stability degree was calculated using the following equation:

$$\text{Stability degree (\%)} = \frac{W_d}{W_o} \times 100,$$

where W_o is the weight of initial air-dried sample and W_d is the weight of the air-dried sample after incubation in PBS. These experiments were done for at least three samples of the same hydrogel.

2.3.4.5 Scanning electron microscopy (SEM)

SEM image were taken using a scanning electron microscope (JEOL LV-5610). Freeze-dried hydrogel sample was put on the stub over a double-sided sticky carbon sheet, and then sputtered for 60 seconds using Denton Vacuum Desk V TSC in vacuum. Then the platinum plated stub was placed inside the scanning electron microscope (JEOL LV-5610) chamber under high vacuum for image taking.

2.3.4.6 In vitro drug release studies

The drug-loaded hydrogels were immersed into 15ml of PBS (pH 4.5, pH 5.5 or pH 7.4) at 37°C. At predetermined time intervals, 1 ml of release medium was withdrawn and estimated by Ultraviolet-visible (UV-vis) spectroscopy at 248 nm for brimonidine tartrate (BT)-loaded sample.

1 mL of fresh PBS, pre-equilibrated at 37°C was immediately added to maintain its volume. The absorbance of the withdrawn solution was measured at each time-point and referenced against the standard curve: $\text{absorbance} = 0.00630 * (\text{concentration in microliter/ml}) + 0.0050$ ⁵⁹ to indicate the cumulative amount (mg) of drug release shown by the following equation:

$$\text{cumulative release percentage}(t) = \frac{C_{tn} * 15(\text{ml}) + \sum_{i=1}^{n-1} C_{ti} * (1\text{ml})}{W_0} * 100\%$$

For timolol maleate, the upmost absorbance wavelength is 294nm, and its reference regression equation is $\text{absorbance} = 0.0186x + 0.0032$ ⁶⁰. These experiments were done for at least three samples of the same hydrogel.

2.3.4.7 Cell culture

Human corneal epithelial cells (HCECs), purchased from Life Technologies Corporation, are normal human corneal epithelial cells isolated from dissected limbal sections, the progenitor-rich region where the sclera and cornea join. HCEC cells were cultured in Keratinocyte SFM at 37°C in a humidified atmosphere containing 5% CO₂ for 5 or 7 days or until 80%-90% confluent.

2.3.4.8 WST-1 assay

Cytotoxicity of G3 hydrogel was evaluated. 1mg of G3 hydrogel was immersed into 1ml of cell culture medium for 24 hours to gain degradation solution. HCECs were seeded in a 96-well cell culture plate at a density of 5×10^3 cells per well. After 48 hours of culture in Keratinocyte SFM at 37°C in a humidified atmosphere containing 5% CO₂, cell culture medium was extracted and hydrogel degradation solution was added to sample groups. Untreated HCECs were used as

control group. The cell viability was assessed with WST-1. These experiments were done for at least three samples of the same hydrogel.

2.3.4.9 ELISA

G3 hydrogel was placed in cell culture medium for HCECs for 24 hours to gain hydrogel degradation solution. 200 μ L of hydrogel degradation solution was added to treat HCECs as sample groups. After 48-hour incubation in Keratinocyte SFM at 37°C in a humidified atmosphere containing 5% CO₂, cell culture medium was collected and used for ELISA using Human IL-1 α ELISA kit by Thermo Scientific. These experiments were done for at least three samples of the same hydrogel.

2.3.4.10 Statistical analysis

Statistical analysis was performed with Prism software. Most of the statistical analysis was based on one way analysis of variance (ANOVA: Single Factor) and Tukey's multiple comparison test. T-test was employed when appropriate. A p-value less than 0.05 was considered statistically significant for rejecting the null hypothesis. Recording data was done in Microsoft Excel 2012. Analysis of data and its graphical representation were done on Prism software, where the error bars represent standard deviations.

CHAPTER 3 RESULTS AND DISCUSSION

3.1 Characterization of G3-alkyne conjugates

Alkyne-PEG₅-acid was conjugated to G3 in the presence of DMTMM. The amine group was highly reactive towards activated carboxyl. Activated carboxyl and amine groups formed an amide linkage between alkyne-PEG₅-acid and G3. The conjugation of alkyne-PEG₅-acid and G3 was confirmed by ¹H-NMR. In the ¹H-NMR spectrum of G3-alkyne conjugate (Figure 3-1), the PEG proton peak (δ 3.71 ppm), multiple proton peaks of G3 (δ 2.35-3.40 ppm). Corresponding peak area was integrated to determine the average number of alkyne per G3-alkyne conjugate has. The results indicated that 66.3% surface groups of G3 dendrimer had been modified with alkyne groups. The molecular weight of G3-alkyne conjugate was calculated to be 13007gmol⁻¹.

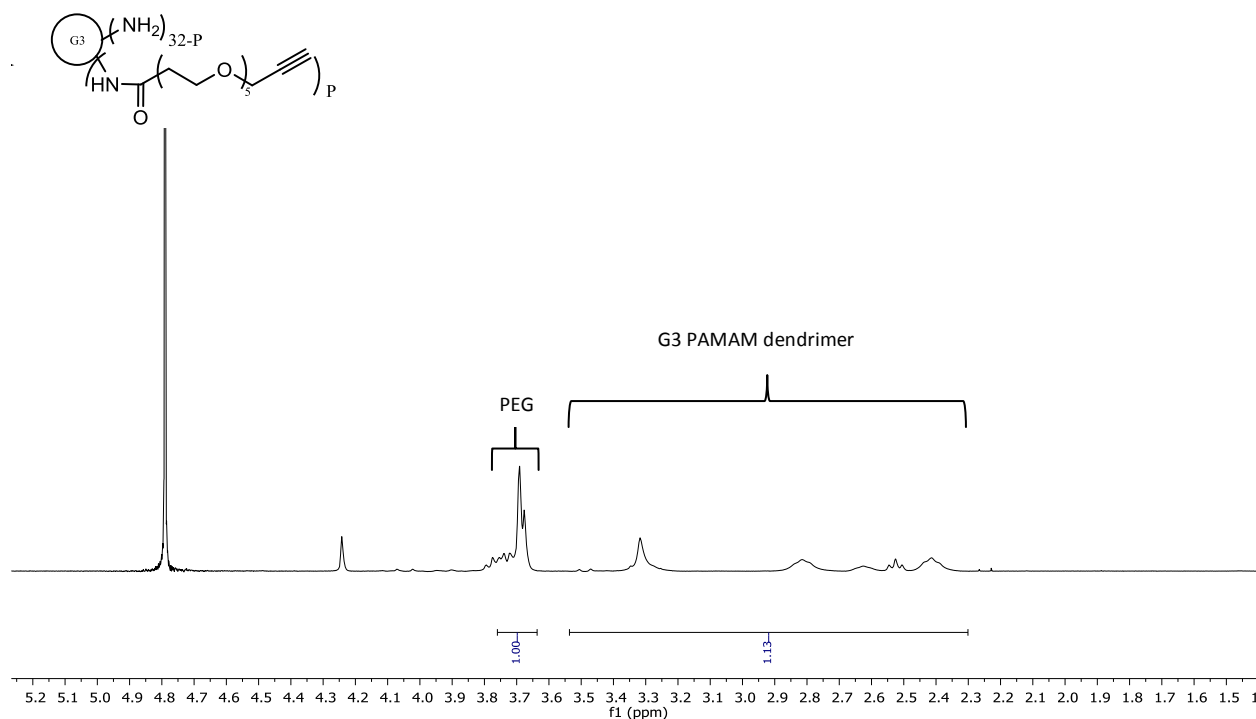


Figure 3-1 ¹H NMR (300 MHz) spectrum of G3-alkyne conjugates in D₂O.

3.2 Characterization of G4.5-alkyne conjugates

Propargylamine was conjugated to G4.5 in the presence of DMTMM to form an amide linkage between propargylamine and G4.5. The conjugation was confirmed by ^1H -NMR after a further reaction with azide-PEG5-acid. The resultant compound was analyzed by using ^1H -NMR (Figure 3-2). The PEG proton peak (δ 3.71 ppm), multiple proton peaks of G4.5 (δ 2.35-3.40 ppm) and single proton peak of the single proton on the triazole (δ 6.14 ppm) indicated the success of the synthesis of G4.5-alkyne conjugates. Corresponding peak area was integrated to determine the average number of alkyne per G4.5-alkyne conjugate has. The results indicated that 7.7% surface groups of G4.5 dendrimer had been modified with alkyne groups. The molecular weight of G4.5-alkyne conjugate was calculated to be $23663\text{g}\cdot\text{mol}^{-1}$.

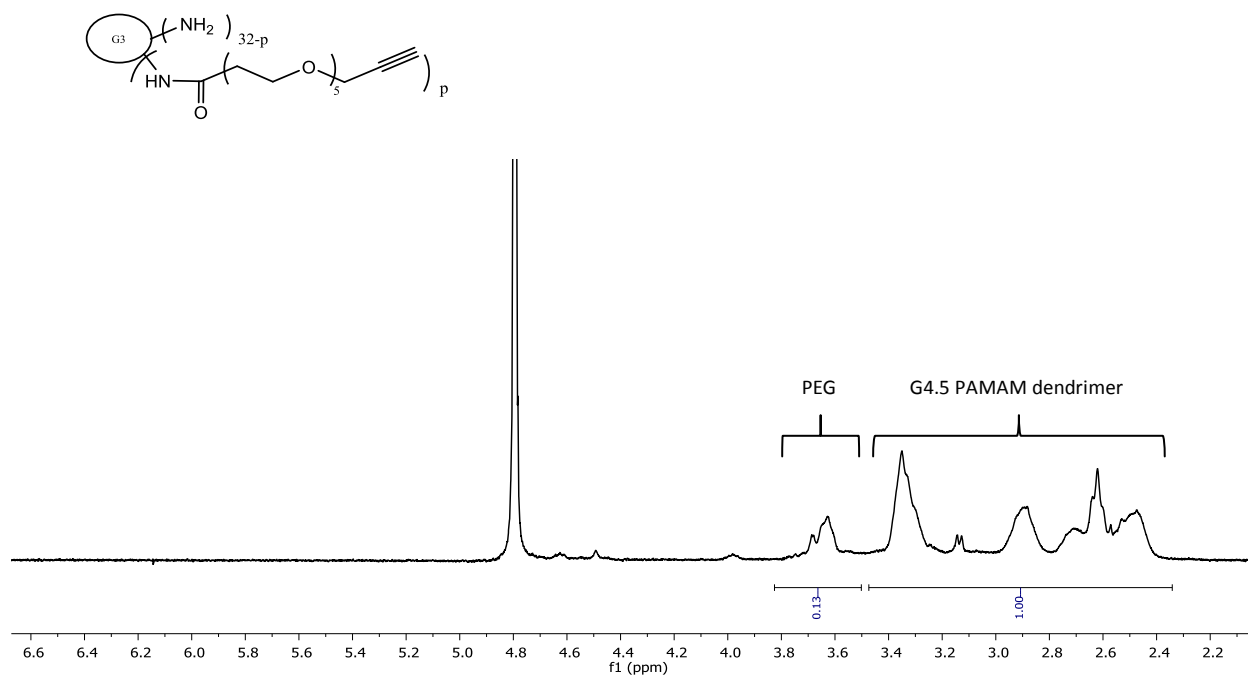


Figure 3-2 ^1H NMR (300 MHz) spectrum of G4.5-alkyne conjugates in D_2O .

3.3 Formation of G3 and G4.5 hydrogels and anti-glaucoma drugs encapsulation

Procedures of G3 hydrogel formation and drug encapsulation are shown in Figure 3-3. G4.5 hydrogel formation is shown in Figure 3-4. Hydrogel without drug loading was formed in vial 1. Hydrogel loaded with timolol maleate was formed in vial 2. Hydrogel loaded with brimonidine tartrate was formed in vial 3.

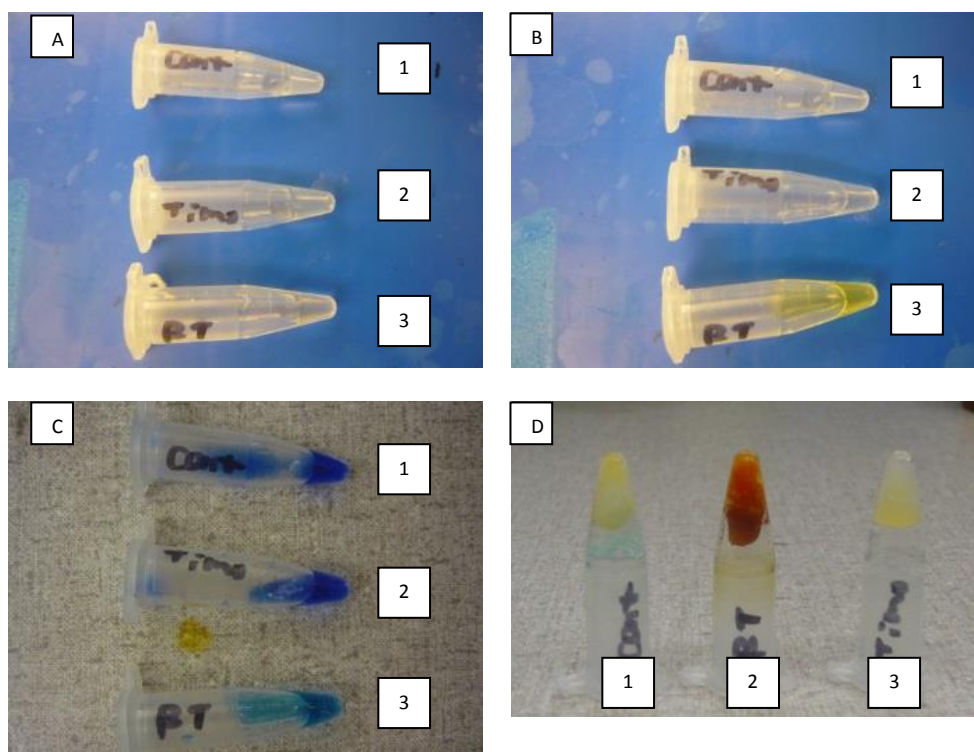


Figure 3-3 G3 hydrogel formation and drug encapsulation. A: transparent solution of PEG-BA and G3-alkyne in 200 μl DI water. B: 4mg of timolol maleate added to vial 2 and 4mg of brimonidine tartrate added to vial 3. C: reaction solutions turned blue after $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added. D: hydrogel formed immediately after sodium ascorbate added and stirred. The molar ratio of alkyne: azide: Cu: sodium ascorbate was 1: 1.5: 0.5: 1.



Figure 3-4 G4.5 hydrogel formation. A: transparent solution of PEG-BA and G4.5-alkyne in 200 µl DI water. B: Reaction solutions turned blue after $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ added. D: Hydrogel formed immediately after sodium ascorbate added and stirred. The molar of alkyne: azide: Cu: sodium ascorbate was 1: 1.5: 0.5: 1.

3.4 G3 hydrogel mechanical property

The mechanical properties of G3 hydrogel were tested using the MTS Bionix 200 ® Mechanical Testing System in conjunction with TestWorks 4.0 software. The scaffolds mechanical properties of three air-dried G3 hydrogel samples including peak load, peak stress and modulus were obtained (Table 3-1).

Table 3-1 Mechanical property of G3 air-dried hydrogel

Specimen #	Width (mm)	Thickness (mm)	Peak Load (N)	Peak Stress (MPa)	Modulus (MPa)
1	1.15	1.47	0.412	0.2	8.032
2	1.09	1.41	0.457	0.3	7.797
3	1.11	1.58	0.771	0.4	10.359
Mean	0.12	1.49	0.547	0.3	8.729
SD	0.03	0.09	0.196	0.1	1.416

3.5 G3 hydrogel swelling property

The swelling property of the hydrogels was examined. As shown in Figure 3-5 and Figure 3-6, swelling degree quickly increased at an average speed of 4.343% per minute in the first 70 minutes. Afterwards until 580 minutes, swelling degree increased slowly with an average speed of 0.0765% per minute. At 1640 minute, G3 hydrogel displayed the maximal swelling degree of 349%.

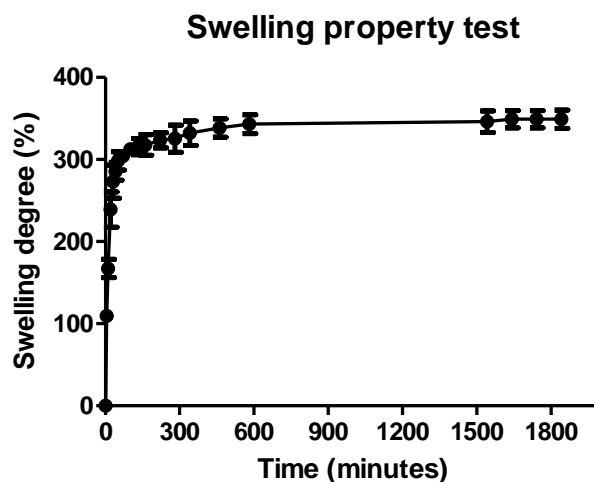


Figure 3-5 G3 hydrogel swelling property tested in DI water for about 1800 minutes at room temperature.

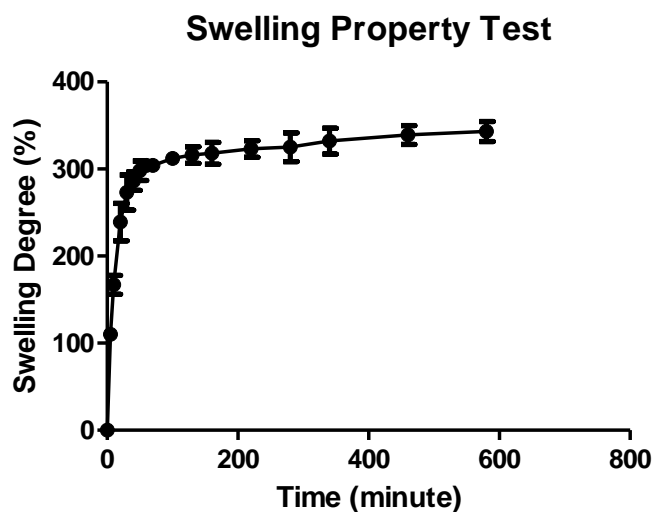


Figure 3-6 G3 hydrogel swelling property tested DI water in the first 600 minutes at room temperature.

3.6 G3 hydrogel pH-dependent degradation study

In vitro degradation studies were conducted to evaluate the hydrogel's stability in PBS of three different pHs (4.5 5.5 7.4) at 37°C. As samples immersed in PBS, they began to swell immediately. Figure 3-7 shows that no degradation was shown after samples immersed in pH 7.4 PBS for all 5 days. As for samples immersed in pH 4.5 and pH 5.5 PBS, the degradation rate was rather slow. The hydrogel immersed in pH 4.5 PBS showed no degradation in the first 3 days at all. On the fourth day, it lost 3.0% of its initial weight and showed no further degradation on the fifth day. As for the hydrogel tested in the pH 5.5 PBS, it showed no degradation in the first 4 days, and on the fifth day, it lost 2.6% of its initial weight. No significant statistical differences ($p < 0.05$) were shown between samples evaluated in all media.

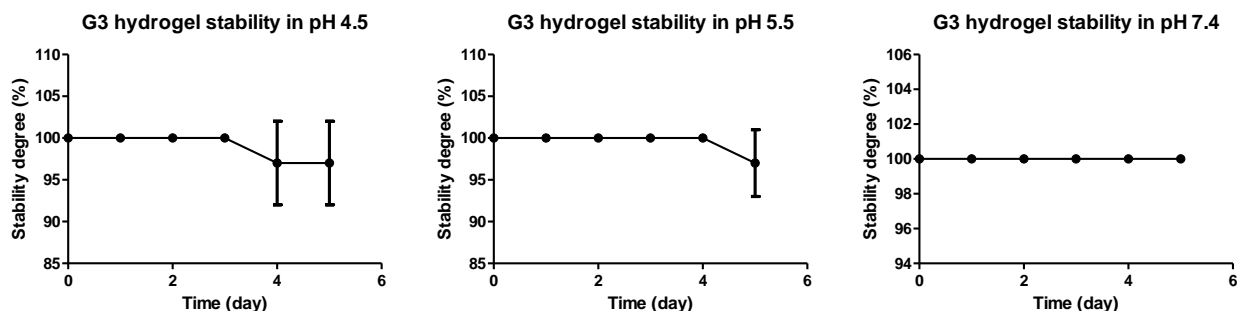


Figure 3-7 G3 hydrogel pH-dependent degradation at 37°C.

3.7 Scanning electronic microscopy (SEM)

Scanning electron microscopy was applied to exam micro structure of drug-loaded hydrogel before and after dialysis. SEM micrographs of the drug-loaded and purified G3 and G4.5 hydrogels are presented in Figure 3-8 and Figure 3-9 respectively. The hydrogel samples possessed an interconnected porous structure with various pore sizes from submicrometers to a

few micrometers. After dialysis, the hydrogels had larger micropores than those drug-loaded samples. Drug particles can be clearly observed in drug-loaded hydrogels. After dialysis, no visible drug particles can be seen in the hydrogel due to drug release.

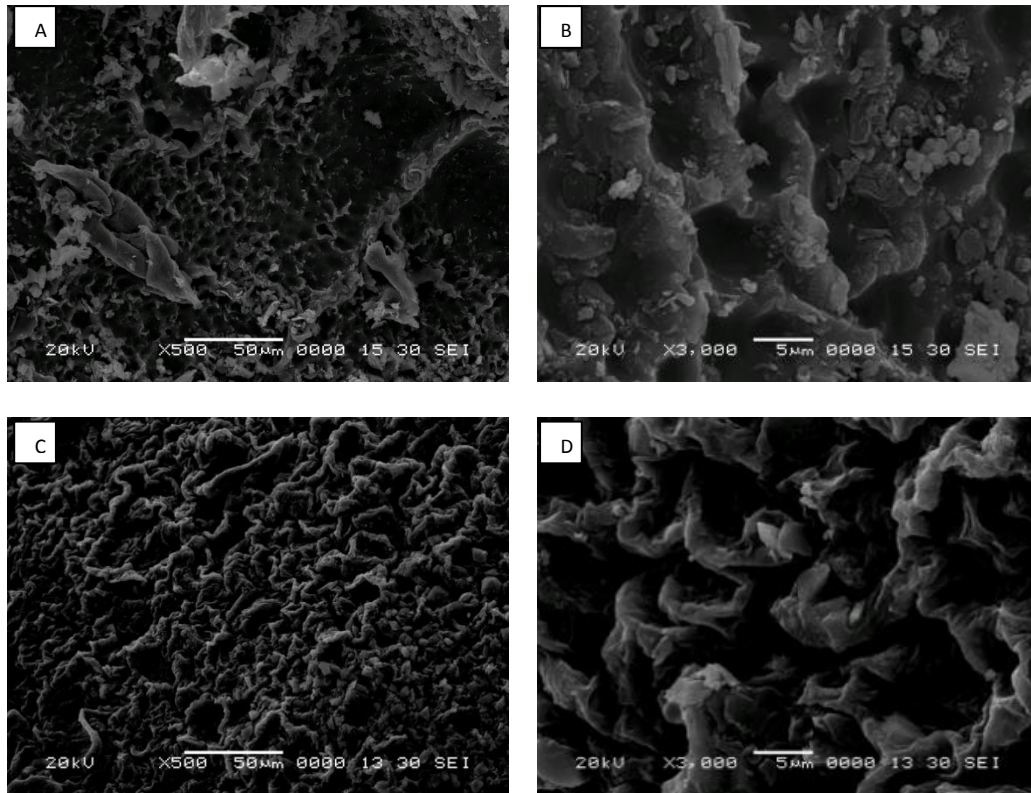


Figure 3-8 SEM images of G3 hydrogels containing drugs before purification (A and B) and after purification(C and D).

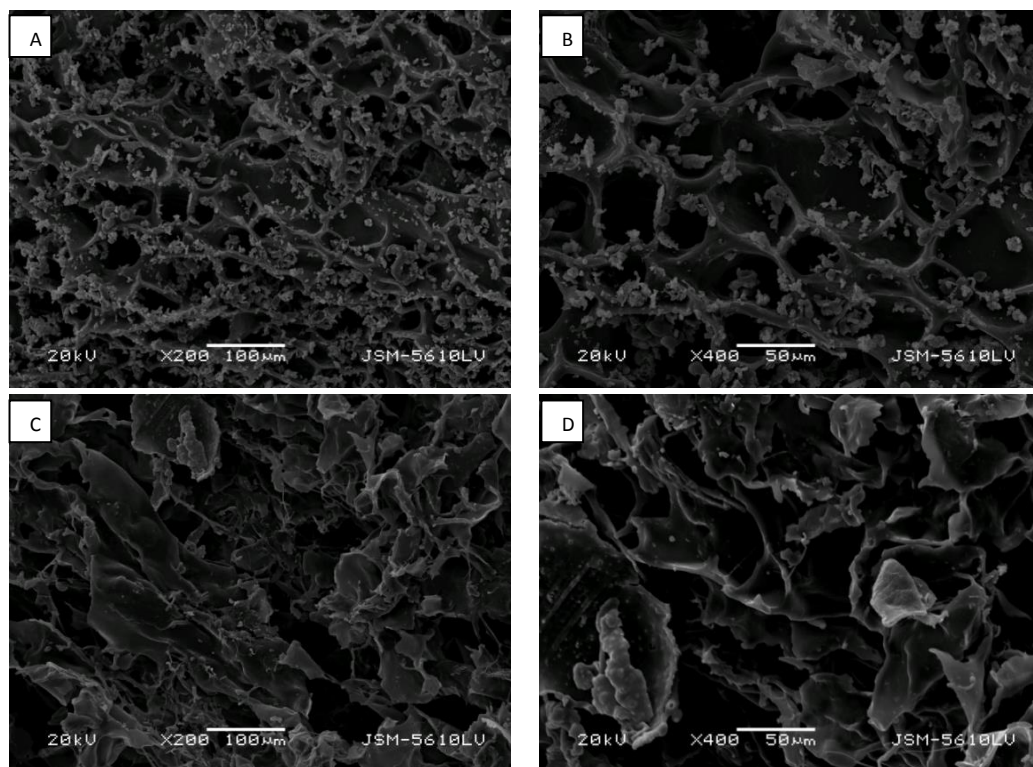


Figure 3-9 SEM images of G4.5 hydrogels containing drugs before purification (A and B) and after purification (C and D).

3.8 Brimonidine tartrate and timolol maleate release from G3 hydrogel

Brimonidine tartrate and timolol maleate release were measured in PBS of three pHs (4.5, 5.5 and 7.4) at 37°C. As shown in Figure 3-10, 64.33% of brimonidine tartrate was released in pH 4.5 PBS, 65.35% was released in pH 5.5 PBS, 72.48% was released in pH 7.4 PBS in 15 days. Most of the drugs were release in the first 16 hours. Data analysis shows that no significant difference in brimonidine tartrate releasing was observed between the three groups.

As shown in Figure 3-11, 60.06% of timolol maleate was released in pH 4.5 PBS, 53.44% was released in pH 5.5 PBS and 74.65% was released in pH 7.4 PBS over a period of 15 days. Data analysis shows that timolol maleate release profile in pH 4.5 PBS and pH 5.5 PBS were not

significantly different. A significantly higher drug release profile was observed in pH 7.4 PBS than in pH 4.5 PBS and pH 5.5 PBS.

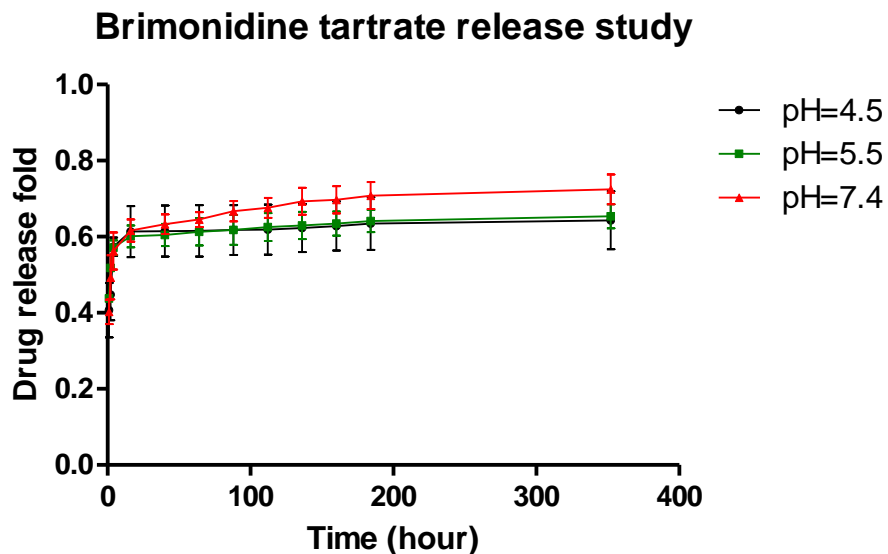


Figure 3-10 Kinetics of brimonidine tartrate release from G3 hydrogel at 37°C.

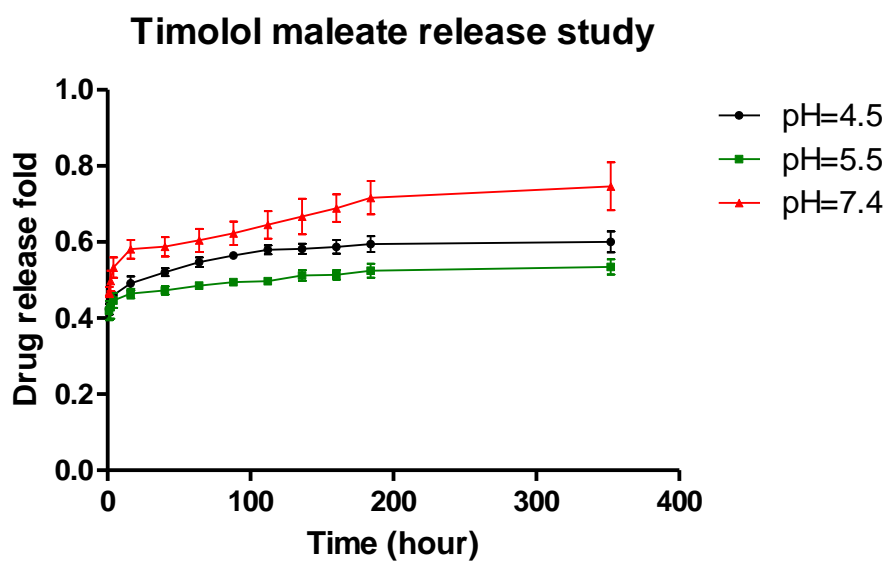


Figure 3-11 Kinetics of timolol maleate release from G3 hydrogel at 37°C.

3.9 G3 hydrogel WST-1 assay

The cytotoxicity was evaluated using human corneal epithelial cells (HCECs). Untreated HCECs were used as a positive control. As seen in Figure 3-12, there was no significant difference between the sample groups and control group in terms of viability. G3 hydrogel degradation solution did not show cytotoxicity effect on HCECs. This non-toxicity effect may be the result of slow degradation of the hydrogel in aqueous solutions.

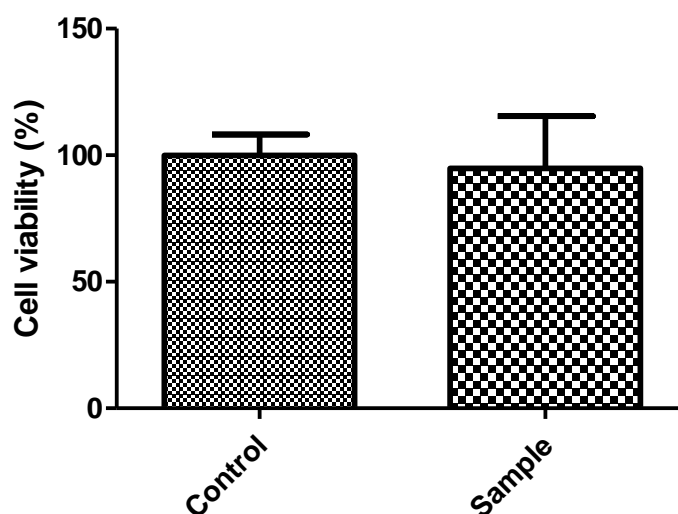


Figure 3-12 Cytotoxicity of G3 hydrogel degradation solution on HCECs.

3.10 G3 hydrogel ELISA

An ELISA kit was used to measure IL-1 α in culture supernatants, an indicator of inflammation. As shown in Figure 3-13 and Figure 3-14, there was no significant difference in IL-1 α release level between control groups and sample groups treated with G3 hydrogel degradation solution. The ELISA result indicated that G3 hydrogel did not induce inflammation in HCECs.

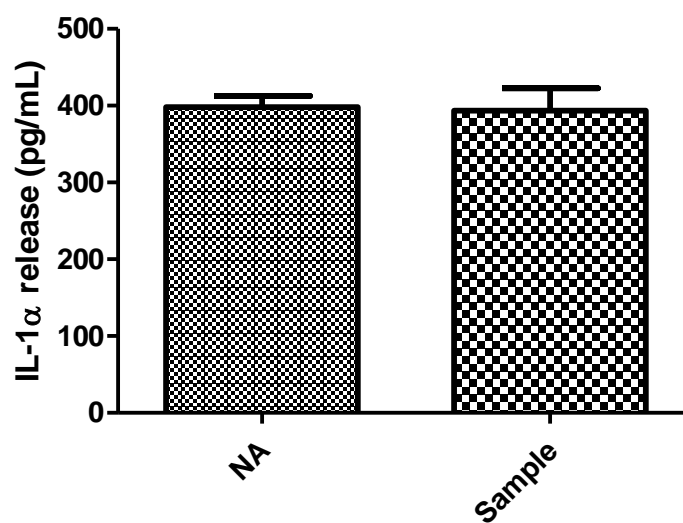


Figure 3-13 Comparison of IL-1 α release from HCECs between control group and sample group treated with G3 hydrogel degradation solution.

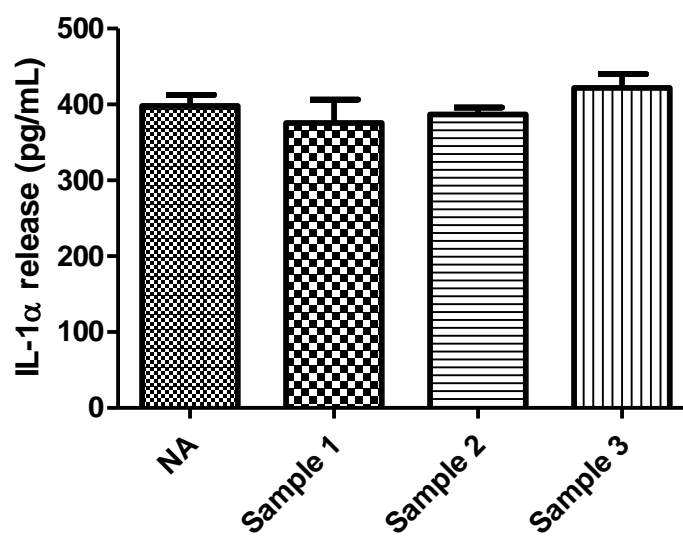


Figure 3-14 Comparison of IL-1 α release from HCECs between control group and each sample group treated with G3 hydrogel degradation solution.

CHAPTER 4 SUMMARY AND FUTURE WORK

A novel clickable hydrogel utilizing G3 or 4.5 PAMAM dendrimer was synthesized and characterized for anti-glaucoma drug delivery. We demonstrated that the G3 hydrogel had no significant degradation and showed a prolonged drug release profile for anti-glaucoma drugs: brimonidine tartrate and timolol maleate. We demonstrated G3 hydrogel had good swelling property. Both WST-1 assay and ELISA showed that hydrogel did not cause toxic effect on human corneal epithelial cells in terms of viability and IL-1 α release.

In this formulation, copper ions may cause toxicity to cells by destructing cells' metabolic pathway and preventing proliferation. To address it, we suggest two solutions. From drug loading perspective, the formulation could be constructed by soaking purified hydrogel in high-concentration drug solution, in other words, by loading drugs after copper ions' removal. Concerning drugs' solubility, this method may be inefficient in loading lipophilic drugs. Another solution is from synthetic perspective, which is more fundamental by using copper-free click chemistry to synthesize hydrogel. In this way, the use of EDTA for removal of copper ions can be avoided. It also helps maintain hydrogel's original micro morphology and mechanical property.

In this work, in vitro drug release profiles were obtained. Further studies will examine in vivo pharmacokinetics and pharmacodynamics of the delivered drug and conduct safety assessment. Clickable hydrogel can also be explored to deliver a broad spectrum of therapeutics, such as small molecular weight drugs, nucleic acids, peptides and proteins to treat various diseases.

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APPENDIX

1. Swelling property

time(min)	Degree of swelling (%)				
	Sample #1	Sample #2	sample#3	mean	SD
0	0%	0%	0%	0%	0
5	102%	113%	114%	110%	0.06248355
10	168%	156%	178%	167%	0.107891802
20	259%	216%	242%	239%	0.216185365
30	288%	250%	281%	273%	0.200640784
40	298%	281%	278%	286%	0.105631369
50	310%	288%	297%	298%	0.111576096
60	312%	303%	297%	304%	0.075420778
70	312%	303%	297%	304%	0.075420778
100	320%	309%	308%	312%	0.061754183
130	327%	309%	311%	316%	0.096153179
160	332%	313%	308%	318%	0.124674594
220	334%	319%	317%	323%	0.095474834
280	344%	313%	319%	325%	0.164951103
340	349%	322%	325%	332%	0.147149725
460	351%	331%	333%	339%	0.109775308
580	356%	334%	339%	343%	0.114628621
1540	361%	341%	336%	346%	0.13246168
1640	361%	344%	342%	349%	0.105979337
1740	361%	344%	342%	349%	0.105979337
1840	361%	347%	339%	349%	0.111835326

2. Degradation property

time (day)	Remaining weight/initial weight in pH=4.5			Remaining weight/initial weight pH=5.5			Remaining weight/initial weight in pH=7.4		
	#1	#2	#3	#1	#2	#3	#1	#2	#3
0	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1
4	1	0.909091	1	1	1	1	1	1	1
5	1	0.909091	1	1	1	0.923077	1	1	1
Time (day)	Sustainable degree in pH=4.5			Sustainable degree in pH=5.5			Sustainable degree in pH=7.4		
	mean	sd	n	mean	sd	n	mean	sd	n
0	1	0	3	1	0	3	1	0	3
1	1	0	3	1	0	3	1	0	3
2	1	0	3	1	0	3	1	0	3
3	1	0	3	1	0	3	1	0	3
4	0.969697	0.052486	3	1	0	3	1	0	3
5	0.969697	0.052486	3	0.974359	0.044412	3	1	0	3

Parameter
Table Analyzed Data 1

One-way analysis of variance
P value 0.3001
P value summary ns
Are means signif. different? (P < 0.05) No
Number of groups 3
F 1.306
R squared 0.1483

Bartlett's test for equal variances
Bartlett's statistic (corrected)
P value
P value summary ns
Do the variances differ signif. (P < 0.05) No

ANOVA Table SS df MS
Treatment (between columns) 0.0003085 2 0.0001543
Residual (within columns) 0.001772 15 0.0001181
Total 0.002081 17

Tukey's Multiple Comparison Test Mean Diff. q Significant? P < 0.05? Summary 95% CI of diff
PH=4.5 vs PH=5.5 -0.005827 1.313 No ns -0.02213 to 0.01048
PH=4.5 vs PH=7.4 -0.01010 2.276 No ns -0.02640 to 0.006202
PH=5.5 vs PH=7.4 -0.004274 0.9630 No ns -0.02058 to 0.01203

3. Timolol maleate release

time hour	pH=4.5			pH=5.5			pH=7.4		
	#1	#2	#3	#1	#2	#3	#1	#2	#3
1	0.4072	0.4324	0.4311	0.3913	0.4368	0.4311	0.4689	0.4819	0.4554
2	0.4328	0.4404	0.4324	0.3945	0.4485	0.443	0.4961	0.5254	0.472
4	0.4558	0.4664	0.4593	0.4241	0.4582	0.4574	0.5246	0.563	0.5114
16	0.4727	0.4927	0.5089	0.4521	0.4645	0.477	0.5836	0.6043	0.5553
40	0.5087	0.5255	0.5278	0.4675	0.4656	0.4854	0.5952	0.6085	0.5591
64	0.5327	0.5531	0.5556	0.4838	0.4791	0.493	0.6088	0.6322	0.572
88	0.5674	0.5619	0.5654	0.4934	0.4962	0.4937	0.6374	0.644	0.5875
112	0.5907	0.5671	0.5807	0.499	0.4984	0.4943	0.6531	0.6769	0.6059
136	0.5945	0.5683	0.5832	0.528	0.5053	0.503	0.6817	0.7047	0.6148
160	0.6065	0.5714	0.5842	0.5283	0.5055	0.5077	0.7065	0.7131	0.6478
184	0.617	0.5761	0.5896	0.5461	0.5143	0.5143	0.7448	0.7387	0.666
352	0.6309	0.5777	0.5933	0.5549	0.5152	0.5332	0.8053	0.7542	0.68
time hour	pH=4.5			pH=5.5			pH=7.4		
	mean	SD	N	mean	SD	N	mean	SD	N
1	0.423567	0.014189	3	0.419733	0.024788	3	0.468733	0.013251	3
2	0.4352	0.004508	3	0.428667	0.029717	3	0.497833	0.026742	3
4	0.4605	0.005401	3	0.446567	0.019461	3	0.533	0.026806	3
16	0.491433	0.018133	3	0.464533	0.01245	3	0.581067	0.024598	3
40	0.520667	0.010427	3	0.472833	0.010924	3	0.5876	0.025562	3
64	0.547133	0.012562	3	0.4853	0.00707	3	0.604333	0.030348	3
88	0.5649	0.002784	3	0.494433	0.001537	3	0.622967	0.030892	3
112	0.5795	0.011846	3	0.497233	0.002558	3	0.6453	0.036137	3

136	0.582	0.013141	3	0.5121	0.013818	3	0.667067	0.046702	3
160	0.587367	0.017763	3	0.513833	0.012577	3	0.689133	0.035948	3
184	0.594233	0.02084	3	0.5249	0.01836	3	0.7165	0.043841	3
352	0.600633	0.027348	3	0.534433	0.019879	3	0.7465	0.063004	3

Parameter
Table Analyzed Data 1

One-way analysis of variance
P value 0.0001
P value summary ***
Are means signif. different? (P < 0.05) Yes
Number of groups 3
F 12.13
R squared 0.4237

Bartlett's test for equal variances
Bartlett's statistic (corrected) 6.613
P value 0.0367
P value summary *
Do the variances differ signif. (P < 0.05) Yes

ANOVA Table SS df MS
Treatment (between columns) 0.1041 2 0.05206
Residual (within columns) 0.1416 33 0.004291
Total 0.2457 35

Tukey's Multiple Comparison Test Mean Diff. q Significant? P < 0.05? Summary 95% CI of diff
PBS PH=4.5 vs PBS PH=5.5 0.04938 2.611 No ns -0.01629 to 0.1151
PBS PH=4.5 vs PBS PH=7.4 -0.08107 4.287 Yes * -0.1467 to -0.01540
PBS PH=5.5 vs PBS PH=7.4 -0.1305 6.899 Yes *** -0.1961 to -0.06478

4. Brimonidine tartrate release

time hour	pH=4.5			pH=5.5			pH=7.4		
	#1	#2	#3	#1	#2	#3	#1	#2	#3
1	0.4896	0.3708	0.3607	0.4335	0.4368	0.4441	0.4386	0.3911	0.3778
2	0.5251	0.419	0.3993	0.5173	0.5124	0.5236	0.5587	0.4708	0.4497
4	0.5998	0.5529	0.5683	0.5709	0.5522	0.5901	0.6148	0.5533	0.5192
16	0.689	0.5614	0.5903	0.5898	0.5798	0.6336	0.648	0.5903	0.611
40	0.69	0.5617	0.5924	0.5935	0.5821	0.6375	0.6604	0.6094	0.6291
64	0.6914	0.5622	0.5934	0.6034	0.5832	0.6527	0.6663	0.6284	0.6406
88	0.6916	0.5665	0.5947	0.6079	0.5851	0.6611	0.6966	0.644	0.6606
112	0.6926	0.5666	0.5976	0.6125	0.5969	0.6664	0.7048	0.653	0.6705
136	0.6947	0.5743	0.5995	0.6242	0.5965	0.6678	0.7324	0.6629	0.6844
160	0.7	0.5746	0.6101	0.6281	0.6064	0.6691	0.7352	0.6643	0.6917
184	0.7121	0.5801	0.6117	0.6329	0.617	0.6736	0.7419	0.671	0.7119
352	0.7284	0.5803	0.6211	0.6501	0.6235	0.6868	0.7627	0.6842	0.7275
Time (hour)	pH=4.5			pH=5.5			pH=7.4		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
1	0.407033	0.071683	3	0.438133	0.005424	3	0.4025	0.031963	3
2	0.4478	0.067665	3	0.517767	0.005615	3	0.493067	0.057811	3
4	0.573667	0.023906	3	0.571067	0.018951	3	0.562433	0.04845	3

16	0.613567	0.066906	3	0.601067	0.028615	3	0.616433	0.029231	3
40	0.6147	0.066994	3	0.604367	0.029255	3	0.632967	0.025719	3
64	0.615667	0.067417	3	0.6131	0.035751	3	0.6451	0.019347	3
88	0.6176	0.065619	3	0.618033	0.039	3	0.667067	0.02689	3
112	0.618933	0.065653	3	0.625267	0.036466	3	0.6761	0.02635	3
136	0.622833	0.063501	3	0.6295	0.035944	3	0.693233	0.035582	3
160	0.628233	0.064637	3	0.634533	0.031841	3	0.697067	0.035753	3
184	0.634633	0.068924	3	0.641167	0.029191	3	0.708267	0.035589	3
352	0.643267	0.076498	3	0.653467	0.031784	3	0.7248	0.03932	3

Parameter

Table Analyzed Data 1

One-way analysis of variance

P value 0.4411

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0.8390

R squared 0.04839

Bartlett's test for equal variances

Bartlett's statistic (corrected) 2.146

P value 0.3419

P value summary ns

Do the variances differ signif. (P < 0.05) No

ANOVA Table SS df MS

Treatment (between columns) 0.01060 2 0.005299

Residual (within columns) 0.2084 33 0.006316

Total 0.2190 35

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary 95% CI of diff
PBS PH=4.5 vs PBS PH=5.5	-0.009128	0.3979	No	ns -0.08880 to 0.07054
PBS PH=4.5 vs PBS PH=7.4	-0.04009	1.748	No	ns -0.1198 to 0.03958
PBS PH=5.5 vs PBS PH=7.4	-0.03096	1.350	No	ns -0.1106 to 0.04871

5. WST-1

Analysis method: t test

Table Analyzed

Column A

vs

Column B

Data 1

Control

vs

Sample

Unpaired t test

P value 0.6178

P value summary ns

Are means signif. different? (P < 0.05) No

One- or two-tailed P value? Two-tailed

t, df t=0.5190 df=8

How big is the difference?

Mean \pm SEM of column A 100.0 \pm 3.644 N=5

Mean \pm SEM of column B 94.85 \pm 9.230 N=5

Difference between means 5.150 \pm 9.923

95% confidence interval -17.73 to 28.03

R squared 0.03257

F test to compare variances

F,DFn, Dfd 6.415, 4, 4

P value 0.0993

P value summary ns

Are variances significantly different? No

6. ELISA result (two groups)

Table Analyzed Data 1

Column A NA

vs vs

Column B Sample

Unpaired t test

P value 0.7378

P value summary ns

Are means signif. different? (P < 0.05) No

One- or two-tailed P value? Two-tailed

t, df t=0.3406 df=16

How big is the difference?

Mean \pm SEM of column A 398.3 \pm 6.485 N=5

Mean \pm SEM of column B 393.6 \pm 8.071 N=13

Difference between means 4.700 \pm 13.80

95% confidence interval -24.56 to 33.96

R squared 0.007198

F test to compare variances

F,DFn, Dfd 4.028, 12, 4

P value 0.1896

P value summary ns

Are variances significantly different? No

7. ELISA (four groups)

One-way analysis of variance

P value 0.0282

P value summary *

Are means signif. different? (P < 0.05) Yes

Number of groups 4

F 4.077

R squared 0.4663

ANOVA Table	SS	df	MS
Treatment (between columns)	5069	3	1690
Residual (within columns)	5801	14	414.4
Total	10870	17	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary 95% CI of diff
NA vs Sample 1	22.50	2.472	No ns	-14.93 to 59.93
NA vs Sample 2	11.30	1.170	No ns	-28.40 to 51.00
NA vs Sample 3	-23.70	2.454	No ns	-63.40 to 16.00